INFECTION AND IMMUNITY, July 1994, p. 2715–2721
0019-9567/94/$04.00+0

Vol. 62, No. 7

Gram-Positive Cell Walls Stimulate Synthesis of Tumor Necrosis Factor Alpha and Interleukin-6 by Human Monocytes

D. HEUMANN,[1] C. BARRAS,[1] A. SEVERIN,[2] M. P. GLAUSER,[1] AND A. TOMASZ[2]*

Division of Infectious Diseases, Department of Internal Medicine, Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland,[1] and Laboratory of Microbiology, The Rockefeller University, New York, New York 10021[2]

Received 27 December 1993/Returned for modification 15 March 1994/Accepted 6 April 1994

Purified cell walls representing a wide variety in teichoic acid and peptidoglycan structure prepared from eight different gram-positive bacterial species induced the production of tumor necrosis factor alpha (TNF-α) and interleukin-6 from human monocytes in the presence of 10% plasma or serum. Significant amounts of cytokines began to be produced at concentrations above 100 ng to 1 μg of cell walls per ml, with maximal production requiring 10 to 100 μg of cell wall material per ml. In the absence of plasma, the cytokine-inducing capacity of cell wall preparations was lower by at least an order of magnitude. The serum-derived cofactor was inactivated by heating at 90°C for 30 min, suggesting that the activity is associated with a protein. On the other hand, replacement of normal with hypogammaglobulinemic plasma, inactivation of complement (at 56°C), and blockade by the monoclonal antibody MY4 of the CD14 receptors on monocytes did not inhibit the production of TNF-α induced by whole cell walls. Cell walls also stimulated production of TNF-α in the presence of polymyxin B, and macrophages derived from the lipopolysaccharide-insensitive cell line of C3He/HeJ mice also produced this cytokine when stimulated by cell walls. Both peptidoglycan and the soluble glycan-teichoic acid component prepared by an enzymatic method from the same wall preparation exhibited a serum-dependent induction of TNF-α from monocytes, while stem peptides and disacharride peptides had only poor, if any, activity. Cell walls may contribute to the septic shock induced by gram-positive bacteria.

Bacterial endotoxin (lipopolysaccharide [LPS]) is known to be primarily responsible for septic shock produced both in humans and in animal models in gram-negative bacterial infections. There is also general agreement that the gram-negative septic shock syndrome involves excessive activation of host macrophages by LPS, resulting in the overproduction of cytokines, especially tumor necrosis factor alpha (TNF-α), interleukin-1 (IL-1), and IL-6 (9, 33).

Much less information is available concerning the microbial factors that are involved in the induction of septic shock by gram-positive bacteria, i.e., microbes that do not produce LPS (4, 5, 21). Clinical studies indicate the presence of metabolic and cardiovascular dysfunctions that are very similar in gram-negative and gram-positive infections (35, 36). *Staphylococcus aureus* and *Staphylococcus epidermidis* were shown to be able to induce a shock-like syndrome in rabbits and dogs without an apparent LPS involvement (26, 34). Moreover, TNF-α was found to mediate the lethality induced not only by inactivated gram-negative bacteria but also by heat-killed gram-positive cells in mice sensitized with galactosamine (10).

In this study, we examined the capacity of highly purified cell walls prepared from eight different species of gram-positive bacteria and the capacity of several cell wall subcomponents to induce the production of TNF-α and IL-6 by human monocytes. The bacterial species were selected to represent major human pathogens and/or chemical variants of the basic gram-positive cell wall structure.

MATERIALS AND METHODS

TNF-α and IL-6 assays. Human peripheral blood mononuclear cells (PBMC) were isolated from normal blood by using Ficoll-Hypaque (LymphoPak; Nycamed, Oslo, Norway) gradients. PBMC (0.5×10^6 cells per ml) were cultivated in microtiter wells (200 μl of cell suspension per well) in RPMI 1640 medium (GIBCO Laboratories, Basel, Switzerland) with or without 10% human plasma (with heparin as an anticoagulant) and with various concentrations of cell walls at 37°C for 4 h (for the assay of TNF-α) and 24 h (for the assay of IL-6). In additional experiments, whole heparinized human blood (200 μl per well), instead of PBMC, was used. Cytokines were measured by bioassays in the supernatant: WEHI clone 13 cells were used for the quantitation of TNF-α, and 7 TD cells were used for assaying IL-6 (1). The sensitivity of the assays is about 25 pg for each cytokine. The specificity of the assay was tested with a murein monoclonal antibody [$F(ab')_2$ fragment] against human TNF-α (provided by M. Kaul, A. G. Knoll Biochemicals, Ludwigshafen, Germany). C3He/HeJ mice were purchased from Jackson Laboratories, Bar Harbor, Maine. Macrophages were obtained by in vitro differentiation of bone marrow precursor cells. Day 10 to 11 macrophages were detached by pipetting, suspended in Dulbecco's modified Eagle's medium (DMEM; Seromed, Munich, Germany), and distributed in 96-well microculture plates (100,000 per well) as described by Betz-Corradin et al. (2).

Inactivation of complement. Inactivation of complement was done by heating the plasma at 56°C for 1 h. Polymyxin B (Sigma Chemical Co., St. Louis, Mo.) at 10 μg/ml was added to the cell walls 10 min before addition to PBMC. Monoclonal antibody MY4 directed against the CD14 antigen of monocytes was obtained from Coulter Immunology (Luton, United Kingdom) (13). MY4 was preincubated with PBMC at 10 μg/ml at 37°C for 30 min prior to the addition of cell walls or LPS. LPS from *Escherichia coli* O111:B4 was purchased from Sigma. Detection of LPS contamination in the wall preparations was by the *Limulus* amoebocyte assay (Kabi Vitrum, Uppsala, Sweden). The source of human plasma was either a plasma pool or autologous plasma from various PBMC donors. Cere-

* Corresponding author. Mailing address: Laboratory of Microbiology, The Rockefeller University, 1230 York Ave., New York, NY 10021. Phone: (212) 327-8277. Fax: (212) 327-8688.

brospinal fluid of rabbits was collected from animals via intracisternal puncture either before or 6 h after the initiation of inflammation by live pneumococci (7).

Bacterial strains and their cultivation. Bacterial strains were selected to represent known variations in the chemical structure of cell walls. These included pneumococci with choline or ethanolamine in their teichoic acid (15), penicillin-resistant pneumococci with indirectly cross-linked peptidoglycan (11), staphylococci with peptidoglycan containing extensively cross-linked pentapeptide cross bridges (8) and ribitol teichoic acids, *Bacillus subtilis* with polyglycerophosphate teichoic acid chains, and *Micrococcus lysodeikticus* walls in which teichoic acid chains are replaced by a succinylated mannan.

Liquid cultures (1 liter) of *B. subtilis* 168, *S. aureus* 209P, COL, and RUSA 208 (an insertional mutant of COL), *S. epidermidis* SUT, and *M. lysodeikticus* were grown at 37°C with vigorous aeration either in Penassay broth (*B. subtilis* and *M. lysodeikticus*) or in tryptic soy broth (staphylococcal strains) (both media were purchased from Difco Laboratories, Detroit, Mich.). Cultures of *Streptococcus sanguis* (Wicky), *Streptococcus pyogenes* T4/56 (group A), the group B streptococcal strain 090R, *Streptococcus pneumoniae* R36A, and its penicillin-resistant genetic transformant *S. pneumoniae* pen 6 (constructed by introducing the penicillin resistance genes of the South African strain 8249 into strain R36A via genetic transformation), were all grown at 37°C without aeration either in Todd-Hewitt broth supplemented with 0.5% glucose (all streptococci except *S. pneumoniae*) or, in the case of pneumococci, in a casein hydrolysate-based synthetic medium (20) supplemented with yeast extract (0.1% [wt/vol] final concentration). In some experiments, the pneumococcal strains were grown in a chemically defined medium (32a) either with choline (5 μg/ml) or ethanolamine (40 μg/ml) as the amino alcohol component.

Preparation of cell walls. For cell wall preparation, a common procedure (adapted from references 8 and 11) was used for all strains. When the optical density at 620 nm of the bacterial culture reached the value characteristic of the mid-logarithmic phase, the culture was quickly chilled in an ice-ethanol bath until the temperature dropped to 4°C. The cells were harvested by centrifugation for 10 min at 16,000 × *g* (4°C). Cells were suspended in saline, and the suspension was quickly dropped into a boiling sodium dodecyl sulfate (SDS) solution at a final concentration of 4%. After 30 min, the denatured cells were collected by centrifugation and washed with several changes of water by centrifugation (30,000 × *g*) at room temperature. The cells were suspended in water and broken immediately with acid-washed glass beads (diameter, 0.2 mm) in a Vortex mixer at maximal speed at 4°C for 15 to 30 min. Unbroken cells and glass beads were removed by low-speed centrifugation (2,000 × *g*), and the cell walls were sedimented at 40,000 × *g* (30 min). The pellet was then resuspended in 100 mM Tris-HCl (pH 7.5) and treated with a sequence of reagents, namely, DNase (EC 3.1.21.1; 10 μg/ml) and RNase (EC 3.1.27.5; 50 μg/ml) in the presence of 20 mM $MgSO_4$ for 2 h and then trypsin (EC 3.4.21.4; 100 μg/ml) in the presence of 10 mM $CaCl_2$ for 16 h. Enzymes were inactivated with boiling 1% SDS (15 min) after which the walls were sedimented and washed with water at room temperature (40,000 × *g*) to remove the SDS. The cell walls were then extracted, in sequence, with 8 M LiCl and 100 mM EDTA, washed twice with water, extracted with acetone, washed three times with distilled water, lyophilized, and stored in the dry state at room temperature.

Amino acid and amino sugar analyses. Samples (4 to 20 nmol) were hydrolyzed in evacuated and sealed glass tubes.

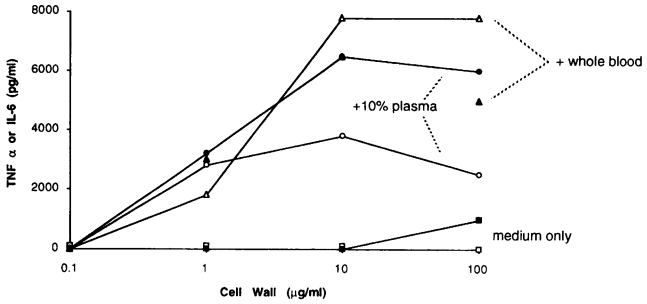

FIG. 1. Effect of plasma on the production of cytokines by monocytes stimulated by gram-positive cell wall preparations. Human PBMC cultured either in medium or in medium supplemented with 10% human plasma or whole human blood were stimulated with various concentrations of *B. subtilis* cell walls as described in Materials and Methods. TNF-α (solid symbols) was measured after 4 h and IL-6 (empty symbols) was measured after 24 h of incubation at 37°C by appropriate bioassays.

For amino acid determinations, the hydrolysis was performed with 6 N HCl at 100°C for 18 h. For amino sugars, 4 N HCl and 4 h of hydrolysis were used. In both cases, a Durrum D-500 autoanalyzer was used for quantitation. Amino acids other than those characteristic of the particular peptidoglycan (alanine, lysine, glutamic acid, glycine, and serine) were not detected in the purified wall preparations (limit of detection, <0.1 mmol per mmol of glutamic acid residue).

Purified cell walls were weighed, resuspended by vigorous Vortex treatment in pyrogen-free saline at various concentrations, and then introduced into the test system.

Teichoic acid-free peptidoglyclan was prepared from intact purified cell walls by treatment with hydrofluoric acid at 0°C for 48 h (8). Disaccharide peptides were prepared by enzymatic hydrolysis of peptidoglycan purified from the cell walls of *S. aureus* COL and RUSA 208 by a previously published method (8). Glycan-teichoic acid complex and glycan-free stem peptides were prepared by the treatment of highly purified *S. pneumoniae* cell walls with affinity-purified pneumococcal *N*-acetylmuramyl-L-alanine amidase (12).

RESULTS

Figure 1 shows the powerful stimulatory effect of plasma on TNF-α and IL-6 production by monocytes incubated with various concentrations of cell wall preparation from *B. subtilis*. In medium without plasma or serum, cell wall concentrations of at least 100 μg/ml were necessary to stimulate monocytes for a detectable TNF-α response, and IL-6 production was not detectable even with this high concentration of cell walls in the unsupplemented RPMI 1640 medium. On the other hand, in the presence of 10% plasma or serum, concentrations of cell wall ranging from 100 ng/ml to 1 μg/ml were sufficient to initiate production of both cytokines. Maximum release was achieved at concentrations of about 10 μg/ml or higher. Similar observations were made when whole blood was used in place of monocytes.

Cell wall concentrations needed to elicit maximal cytokine production were substantially higher than the concentrations of LPS (1 to 10 ng/ml) that are known to induce comparable levels of TNF-α and IL-6 (data not shown).

The specificity of the bioassay used for TNF-α production was tested by the addition of murein monoclonal antibody [$F(ab')_2$ fragments] against human TNF-α. Full inhibition was

TABLE 1. Effect of polymyxin B on TNF-α release by human monocytes stimulated with gram-positive cell walls and LPS in medium containing 10% plasma

Cell wall prepn (10 μg/ml)	No. of expts	TNF-α (pg/ml)	
		Without polymyxin B	With polymyxin B
B. subtilis 168	3	7,500 ± 3,700	6,500 ± 2,300
S. aureus COL (methicillin-resistant strain)	2	1,200 ± 100	1,800 ± 1,900
S. aureus RUSA 208	2	1,450 ± 490	2,300 ± 1,270
S. epidermis	1	800	800
S. sanguis Wicky	3	700 ± 400	700 ± 500
M. lysodeikticus	3	3,800 ± 1,900	3,800 ± 2,600
S. pyogenes (group A)	3	300 ± 100	200 ± 100
S. pneumoniae R36A (choline)	3	1,300 ± 1,100	1,100 ± 700
S. pneumoniae R36A (ethanolamine)	2	1,100 ± 280	1,500 ± 420
S. pneumoniae pen 6 LPS	3	700 ± 100	1,100 ± 600
1 ng/ml	3	800 ± 200	0
10 ng/ml	3	1,300 ± 300	0
100 ng/ml	3	1,200 ± 300	400 ± 400

observed when 10 μg of the monoclonal antibody and 10 μg of the *B. subtilis* cell walls per ml were used, confirming that the cytokine produced was TNF-α. The addition of indomethacin (10^{-6}) to the cytokine induction assay did not increase the amounts of TNF-α produced by stimulation of the monocytes with 10 μg of cell wall per ml, suggesting that coinduction of inhibitory prostaglandins did not take place under the conditions of our assay.

A combination of several tests, as follows, indicated that the cytokine induction was not due to LPS contamination. (i) *Limulus* amoebocyte assays (LPS detection limit, 25 pg) performed on 10-μg aliquots of each of the wall preparations indicated either negligible or no detectable LPS contamination.

(ii) The cytokine induction experiments were repeated with cell walls prepared from 10 different gram-positive bacteria, with and without polymyxin B (10 μg/ml; a known inhibitor of LPS [25]) in the incubation medium. Parallel monocyte cultures were stimulated with LPS. Polymyxin B was able to fully inhibit LPS-induced TNF-α release when a stimulus of 1 to 10 ng of LPS per ml was used; the same concentration of polymyxin B decreased TNF-α production only partially when 100 ng of LPS per ml was used. In contrast, polymyxin B treatment had no significant inhibitory effect on the TNF-α levels stimulated by the gram-positive cell wall preparations (Table 1).

(iii) Cell wall preparations were able to induce the production of TNF-α in bone marrow-derived macrophages from C3He/HeJ mice which do not respond to LPS. C3He/HeJ bone marrow-derived macrophages (10^5) were incubated in 250 μl of DMEM medium enriched with 1% normal murine plasma and stimulated with 100 ng of O111 LPS or 10 μg each of two *S. aureus* cell wall preparations per ml overnight at 37°C. The amounts of TNF-α produced by the macrophages stimulated by LPS, *S. aureus* COL, and *S. aureus* RUSA 208 were 20, 1,300, and 1,600 pg/ml, respectively (means of three separate experiments).

(iv) It has been shown that plasma or serum is essential for the production of TNF-α during stimulation of monocytes by low concentrations (<100 pg/ml) of LPS. At least one LPS-binding factor was shown to be involved with the presentation of LPS molecules to CD14 receptors on the monocyte surface, resulting in the increased expression of LPS-inducible genes such as the determinant of TNF-α (14, 30, 39). Antibody-mediated blocking of CD14 was shown to inhibit TNF-α secretion at low doses of LPS (14, 30, 39). To investigate whether the gram-positive cell wall-induced stimulation of cytokine production followed the same pathway, CD14 was blocked with the MY4 antibody in a series of experiments using four selected wall preparations, each tested at three different concentrations (1, 5, and 10 μg/ml). TNF-α was measured after 4 h of incubation. Substantial reduction in TNF-α levels was observed only with one (group A streptococcus) of the four preparations (Table 2). In contrast, the CD14 blockade was able to reduce TNF-α release with 10 ng of LPS per ml but not with 100 ng of LPS per ml, confirming previous observations (14, 30, 39).

Several tests were done to explore the nature of the plasma-derived cofactor. Heating the plasma at 90°C for 30 min inactivated the cofactor, suggesting that it may be a protein. On the other hand, preheating plasma at 56°C to inactivate complement had no detectable effect on the level of cytokine production.

The effect of the serum immunoglobulin G concentration was tested by comparing TNF-α production in 5% pooled human plasma (five donors, 9.4 mg of IgG per ml) with that in 5% plasma from a hypogammaglobulinemic patient (0.05 mg of IgG per ml). When a 10-μg/ml concentration of cell walls from *S. aureus* COL or *S. pneumoniae* R36A was used, the amounts of TNF-α produced after 4 h of incubation in the normal and immunoglobulin G-deficient plasma samples were 3,200 ± 1,450 and 3,400 ± 1,560 pg/ml, respectively, with the staphylococcal wall and 4,240 ± 1,350 and 4,150 ± 1,610 pg/ml,

TABLE 2. Effect of CD14 blockade on TNF-α release by human monocytes stimulated with various concentrations of gram-positive cell wall preparations

Cell wall prepn	TNF-α released (pg/ml)					
	Without anti-CD14 MAb			With anti-CD14 MAb		
	1 μg/ml[a]	5 μg/ml	10 μg/ml	1 μg/ml	5 μg/ml	10 μg/ml
B. subtilis 168	3,325 ± 1,960	4,100 ± 1,950	5,300 ± 2,470	3,830 ± 2,270	4,200 ± 1,650	4,850 ± 2,710
S. pyogenes (group A)	170 ± 110	1,300 ± 700	1,935 ± 1,150	95 ± 65	400 ± 250	805 ± 630
S. pneumoniae R36A (ethanolamine)	1,400 ± 350	2,100 ± 820	2,350 ± 950	900 ± 650	2,200 ± 800	2,150 ± 1,140
S. pneumoniae R36A	900 ± 350	1,600 ± 1,000	2,100 ± 1,440	1,400 ± 660	2,100 ± 1,220	3,000 ± 1,555

[a] Cell wall concentration.

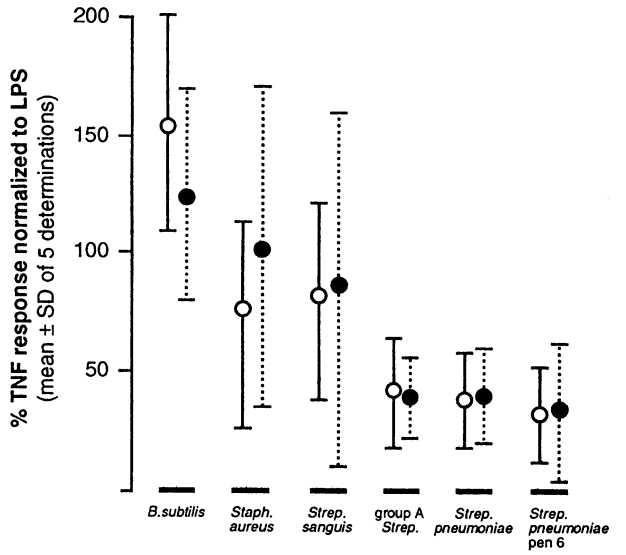

FIG. 2. Percentage of TNF-α (TNF) produced by monocytes of single donors exposed to gram-positive bacterial cell walls. Cell walls prepared from *B. subtilis*, *S. aureus*, *S. sanguis*, group A streptococci, *S. pneumoniae* (penicillin susceptible), and *S. pneumoniae* (penicillin resistant) pen 6, each at a 10-μg/ml concentration, were mixed with monocytes and autologous plasma from the same donor. TNF-α production was determined as described in Materials and Methods. This experiment was repeated with five different monocyte donors and autologous plasma. Monocytes from the same five donors were then combined with a single common source of pooled plasma (20 donors), and the TNF-α induction experiment was repeated with the bacterial cell wall preparations. As an internal control, LPS (O111) at a concentration of 1 ng/ml was included in both the autologous and pooled plasma experiments. In each experiment, the amount of TNF-α produced by stimulation with the LPS control was taken as 100%, and the TNF-α levels produced by the cell wall-stimulated monocytes were normalized to the appropriate LPS-stimulated values. Results are shown as means ± standard deviations (SD) of the five experiments. Symbols: open circles (as means) and solid lines, autologous sera; closed circles (as means) and dashed lines, pooled sera.

respectively, with the pneumococcal wall (means of three experiments ± standard deviations).

To determine whether the different inducing capacities were related to differences between wall preparations, the experiments were repeated with a set of six wall preparations (10 μg/ml each) with monocytes from the same donors in combination with autologous plasma. This test was repeated with five different monocyte sources plus autologous plasma, and the TNF-α-inducing capacity of each wall preparation was expressed as the function of the inducing capacity of LPS (1 ng/ml), which was included in each one of the tests as an internal control for the quality of the monocyte preparation. Figure 2 shows that the specific TNF-α-inducing capacity of *B. subtilis* cell walls was significantly higher ($P < 0.03$ or less in a Student two-tailed t test) than the activity of any one of the other five preparations. When the experiment was repeated with the identical five monocyte preparations but with a common pooled plasma (20 donors) instead of autologous plasma the specific inducing activity of *B. subtilis* was still significantly higher than that of the group A streptococcal preparation or the two pneumococcal preparations ($P < 0.003$). The inducing capacity of *B. subtilis* was still greater than those of *S. aureus* and *S. sanguis* walls, but the differences were no longer significant (data not shown).

To test subcomponents of the cell walls in the cytokine-inducing system, peptidoglycan was prepared from *S. pneumoniae* R36A grown on choline-containing medium and from *S. aureus* COL and RUSA 208. The last strain is a Tn*551*-induced peptidoglycan structural mutant of strain COL (27). High-molecular-weight glycan-teichoic acid complexes with greatly reduced stem peptide contents and glycan- and teichoic acid-free stem peptides were prepared from the pneumococcal cell walls by digestion with the pneumococcal amidase (12). These preparations and the original cell wall preparations were then tested for TNF-α-inducing activity in serum-free medium and also in the presence of 10% pooled plasma with or without decomplementation by treatment at 56°C. The data summarized in Table 3 show that both peptidoglycan and the glycan-teichoic acid complex exhibited TNF-α-inducing activity when tested at 1- and 10-μg/ml concentrations, provided that plasma was present. Inactivation of complement by preheating plasma at 56°C did not reduce the stimulating activity of the plasma factor(s).

TABLE 3. Induction of TNF-α (pg/ml) by human monocytes stimulated by cell wall subcomponents

Bacterial source	Cell wall subcomponent	TNF-α (pg/m) release induced by cell wall prepn with:					
		No plasma		Plasma		Heated plasma[a]	
		1[b]	10	1	10	1	10
S. aureus COL	Wall	0	0	1,600	1,400	1,600	1,800
	PG[c]	20	30	ND[d]	900	ND	1,400
	Muropeptides	0	0	0	400	0	50
S. aureus RUSA 208	Wall	40	500	800	1,200	720	1,800
	PG	100	200	400	1,200	200	800
S. pneumoniae R36A	Wall	0	0	800	3,200	ND	ND
	PG	0	50	200	1,600	ND	ND
	Gly-TA[e]	40	100	400	1,600	100	1,600
	Stem peptides	0	20	0	200	0	50

[a] Plasma was heated to denature complement as described in Materials and Methods.
[b] Cell wall concentration in micrograms per milliliter.
[c] Peptidoglycan.
[d] ND, not done.
[e] Glycan-teichoic acid complex.

DISCUSSION

During the last decade, there has been a major increase in the incidence of bloodstream infections caused by gram-positive bacterial pathogens (4, 5, 21). However, the nature of bacterial components responsible for the initiation of invasive gram-positive disease is not fully understood. The capacity of a variety of protein exotoxins from several gram-positive cocci to trigger the toxic shock syndrome (6, 24, 28) and the production of cytokines is well established (16, 17, 31). On the other hand, cell surface components of gram-positive bacteria may also contribute to the induction of molecular events in disease. Several recent reports in the literature indicate that exploration of this aspect of gram-positive disease has begun in several laboratories. The intravenous injection of high cell equivalent concentrations of *S. epidermidis* (34) and *S. aureus* (22), heat-inactivated to denature inflammatory proteins, caused pathophysiological effects of septic shock and cytokine production in rabbits. However, the cell component responsible for these activities remains unknown. Membrane teichoic acids (lipoteichoic acids) isolated from some gram-positive species were recently shown to induce production of cytokines when incubated with monocytes (3) or bone-marrow-derived mononuclear phagocytes, while other lipoteichoic acid preparations with closely similar chemical structures appeared to be inactive (18). The pneumococcal membrane teichoic acid was claimed to induce production of IL-1 from monocytes (29); however, this finding was not confirmed in another laboratory (18). Pneumococcal cell wall fractions enriched for teichoic acid were reported to elicit production of large amounts of IL-1 but not TNF-α when incubated with monocytes in the absence of serum (29). However, a more recent report (23) has described the ability of both staphylococcal peptidoglycan and teichoic acid (extracted in soluble form from staphylococcal cell walls by cold trichloroacetic acid) to induce both TNF-α and IL-1 and IL-6 from human monocytes under experimental conditions that appear to be similar to the ones used previously (29). These apparently conflicting results may be due to the drastically different chemical compositions of the two teichoic acid preparations used: one (29) was derived from pneumococci containing a complex ribitol teichoic acid which contains phosphoryl choline residues, while the staphylococcal preparation used (23) was ribitol teichoic acid. The production of TNF-α by rabbit peritoneal macrophages stimulated by *S. aureus* peptidoglycan has also been described (19).

At least some of the conflicts in the literature are likely to be related to the different degrees of purity of the preparations used and the differences in the chemical structures of the preparations tested. A particularly suggestive finding in this respect was the observation that extensive sonication of peptidoglycan preparations resulted in the gradual decrease in the specific cytokine-inducing capacity, suggesting the possible need for an intact secondary or tertiary structure in these polymers (32). The superior cytokine-inducing activity of heat-killed staphylococci (compared with the same cell equivalent concentrations of peptidoglycan present in these bacteria) was also interpreted in these terms (32).

For these reasons, it was of particular interest to determine the cytokine-inducing capacity of intact, highly purified cell walls in which the juxtaposition of covalently interlinked wall components (peptidoglycan and teichoic acid chains) should remain unaltered. Our findings clearly show that intact cell walls are capable of induction of TNF-α and IL-6 above the threshold concentration of 0.1 to 1 μg/ml.

The cell wall preparations appeared to be free of LPS as indicated by the negative *Limulus* amoebocyte assay, the lack of effect of polymyxin B on the wall-induced cytokine release, and the continued stimulation of TNF-α production by macrophages derived from the LPS-insensitive macrophage cell line.

The repeated treatment of cell walls during the preparative procedure with protein-denaturing agents and the extraction with solvents with high ionic strengths (divalent cation-complexing agents and organic solvents) make it highly unlikely that the cytokine induction was due to contamination by protein toxins or lipoteichoic acids (both of which are capable of cytokine induction in cells of the monocytic lineage) (3, 16–18, 20). The purity of wall preparations by chemical criteria was provided by chemical analysis and high-performance liquid chromatography analysis of enzymatic hydrolysates in the cases of pneumococcal and staphylococcal cell walls (8, 12).

What subcomponents of the cell wall contribute to the cytokine-inducing activity is not clear. Our findings confirm recent reports (19, 23, 32) describing cytokine-stimulating activity of staphylococcal peptidoglycan and extend it to pneumococcal peptidoglycan as well. We also confirm the observation of Timmerman et al. that stem peptides and disaccharide peptides free of teichoic acid have only greatly reduced or no detectable TNF-α-inducing capacity (32) (Table 3).

On the other hand, the soluble glycan-teichoic acid complexes generated by enzymatic treatment from intact pneumococcal cell walls also had high TNF-α-inducing capacity. Previous work has shown that such preparations contain a mixture of glycan-teichoic acid complexes and a greatly reduced stem peptide content (12).

If one assumes that the cytokine-inducing activity of cell walls described is due primarily to the peptidoglycan component then, as our data suggest, peptidoglycan within an intact cell wall structure must be about two to four times higher in specific activity than peptidoglycan extracted from the intact cell wall (Table 3). Nevertheless, caution should be used in the interpretation of cell wall induction data since heat-denatured staphylococci (which are often considered synonymous with cell walls) appear to have specific cytokine-inducing activity almost 2 orders of magnitude higher than peptidoglycan prepared from the same bacteria (32). One should also remember that, in our experiments (as well as in the majority of studies described in the literature), the cytokines produced were quantitated by bioassays which may underestimate the amounts of cytokines produced because of a possible coinduction of soluble TNF-α or IL-6 receptors. We do not know if such negative regulators appear under the assay conditions used.

The most striking novel observation described in this communication is the requirement for a serum- and/or plasma-derived factor(s) for obtaining an optimal cytokine-stimulating activity for both the intact cell walls and the active subcomponents such as peptidoglycan and the glycan-teichoic acid complex. The data in Fig. 1 and Table 3 and a comparison of them with data in the published literature (23) indicate that the addition of serum or plasma to the assay system increases the specific cytokine-inducing capacity by about 1 order of magnitude. For instance, incubation of monocytes with 10 μg of staphylococcal peptidoglycan per ml resulted in the production of about 0.6 ng of TNF-α per ml (measured in a bioassay) (23), while incubation with intact staphylococcal cell walls at the same concentration and in the presence of 10% plasma or serum resulted in the production of 6.0 ng of TNF-α per ml.

The heat sensitivity of the plasma- and/or serum-derived cofactor(s) suggests that it is a protein. The cofactor does not seem to be complement (37) since the cofactor activity survived the heating of plasma at 56°C. Normal plasma could be

replaced with plasma containing less than 1% of the normal concentration of immunoglobulin G.

With a few exceptions (e.g., group A streptococcal cell wall), the CD14-directed monoclonal antibody MY4 had no effect on the cell wall-stimulated cytokine release, suggesting that the cofactor involved in the case of most cell walls may be distinct from LPS-binding factor (14) or septin (38). This finding agrees with the observation that purified LPS-binding factor had no effect on TNF-α production induced by heat-killed staphylococci or peptidoglycan (22). However, the contribution of the CD14 pathway to cytokine induction by wall subcomponents (peptidoglycan and glycan-teichoic acid complexes) has not yet been tested. Thus, the possibility of some common steps in LPS-induced and wall-induced cytokine induction should be left open.

ACKNOWLEDGMENTS

These investigations received support from a National Institutes of Health grant (AI16794) and a grant from the Swiss National Foundation for Research (32-30265-90).

We would like to acknowledge the stimulating discussions and critique of Philippe Moreillon.

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